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APPLIED AND ENVIRONMENTAL MICROBIOLOGY, APR. 1997, p. 1298-1306
 0099-2240/97/\$04.00+0
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Vol. 63, No. 4

Regulation of Cellulase Gene Expression in the Filamentous Fungus *Trichoderma reesei*

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Received 12 July 1996/Accepted 17 January 1997

Basic features of regulation of expression of the genes encoding the cellulases of the filamentous fungus *Trichoderma reesei* QM9414, the genes *cbb1* and *cbb2* encoding cellobiohydrolases and the genes *egl1*, *egl2* and *egl5* encoding endoglucanases, were studied at the mRNA level. The cellulase genes were coordinately expressed under all conditions studied, with the steady-state mRNA levels of *cbb1* being the highest. Solka floc cellulose and the disaccharide sophorose induced expression to almost the same level. Moderate expression was observed when cellobiose or lactose was used as the carbon source. It was found that glycerol and sorbitol do not promote expression but, unlike glucose, do not inhibit it either, because the addition of 1 to 2 mM sophorose to glycerol or sorbitol cultures provokes high cellulase expression levels. These carbon sources thus provide a useful means to study cellulase regulation without significantly affecting the growth of the fungus. RNA slot blot experiments showed that no expression could be observed on glucose-containing medium and that high glucose levels abolish the inducing effect of sophorose. The results clearly show that distinct and clear-cut mechanisms of induction and glucose repression regulate cellulase expression in an actively growing fungus. However, derepression of cellulase expression occurs without apparent addition of an inducer once glucose has been depleted from the medium. This expression seems not to arise simply from starvation, since the lack of carbon or nitrogen as such is not sufficient to trigger significant expression.

A special feature of filamentous fungi and cellulolytic bacteria is the production of extracellular hydrolytic enzymes which provide them with the capability to use complex plant polysaccharides for growth. The major component of plant material, together with hemicellulose and lignin, is cellulose. It consists of long polymers of β 1,4-linked glucose units, which in turn form higher-order fibrillar structures. Cellulose is to a large extent in a crystalline water-insoluble form in plant material, and its hydrolysis consequently sets a challenge for the saprophytes. The cellulolytic organisms, bacteria and filamentous fungi, produce a set of enzymes which synergistically hydrolyze crystalline cellulose to smaller oligosaccharides and finally to glucose. Because cellulose is the most abundant organic carbon source on Earth, cellulolytic organisms play a very important role in carbon turnover in nature. The cellulolytic capability may also play a significant role in the ability of fungal plant pathogens or antagonistic fungi to attack their target organisms.

One of the most extensively studied cellulolytic organisms is the soft rot fungus *Trichoderma reesei* (reviewed in references 23, 41, and 56). Hypercellulolytic mutant strains secrete large amounts of cellulases, the largest published amounts being about 40 g/liter (9), and *T. reesei* therefore has also considerable industrial importance. The fungus produces a complete set of cellulases that are able to cleave the β 1,4-glycosidic bonds present in cellulose or cellulose derivatives. The cellulases are generally classified as cellobiohydrolases (CBH) (EC 3.2.1.91), which act as exoenzymes and release cellobiose as a main product from crystalline cellulose; endoglucanases (EG) (EC 3.2.1.4), which have high affinity towards soluble cellulose derivatives and attack these by endoaction; and β -glucosidases (EC 3.2.1.21), which hydrolyze cellobiosaccharides and the

disaccharide cellobiose into glucose, providing the fungus with an easily utilized carbon source for growth. Two genes encoding CBHs, *cbb1* (50, 54) and *cbb2* (5, 55), four encoding EGs, *egl1* (38, 60), *egl2* (46), *egl3* (63), and *egl5* (44, 45), and one encoding a β -glucosidase (2, 26) have now been reported from this organism.

The fungus produces high levels of cellulases on media containing cellulose or complex plant material, on cellobionolactone, and, to a variable extent, in the presence of the disaccharides cellobiose or lactose (reviewed in references 4 and 23). Production is not significant when glucose, fructose, or glycerol is used as the carbon source. A high level of cellulase expression is obtained when sophorose, a molecule consisting of two glucose units linked by a β 1,2-linkage, is added into the cultures (28, 37). This molecule could be formed from cellobiosaccharides by the transglycosylation activity of EG1 (3, 8) or β -glucosidase (16, 58).

The fact that the major cellulase genes were cloned by differential hybridization showed that their expression is regulated at transcriptional level, and this has since been confirmed (1, 10-12, 31, 42). However, many basic questions still remain to be thoroughly studied, such as whether the expression of the different cellulases is regulated by a shared mechanism and whether a low level of constitutive expression of cellulases occurs under all conditions. Constitutive expression might be needed for the initial release of a soluble inducing compound from the highly polymeric cellulose, which would further trigger high levels of cellulase expression. It has not been thoroughly studied whether mechanisms of true induction or repression are operating. The biochemical and molecular data available today is to some extent contradictory and has so far provided insufficient data to form the basis for further research directed toward, for instance, analysis of the functional regions in the promoters of the cellulase genes or the regulatory proteins involved. For these reasons, we have attempted to address some of the questions related to cellulase expression in filamentous fungi, and in this article we describe an investiga-

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tion at the mRNA level of the basic mechanisms of cellulase regulation in the filamentous fungus *T. reesei*.

MATERIALS AND METHODS

Strains and vectors. *T. reesei* QM9414 (29) was used throughout the studies. Plasmids pTTC1, pTTC9 (40), pTTc11 (39), pMS1 (46), pAS11 (44, 45) and pSV3 (61) were used as a source of the specific primers for the *cbb1*, *cbb2*, *egf1*, *egf2*, *egf3*, and *pck1* genes, respectively. The *T. reesei* actin gene (30) primer was amplified from chromosomal DNA with sequence-specific primers.

Shake flask cultivations. The minimal medium (MM) used consisted of 3% (lactose) or 2% carbon sources (glucose, cellobiose, sorbitol, or glyceral; when more than one carbon source was used, the concentration of each was 2%), 1.5% KH_2PO_4 , 0.5% $(NH_4)_2SO_4$, 0.005% $MgSO_4$, 0.05% $CuCl_2$, 0.0005% $FeSO_4 \cdot 7H_2O$, 0.00016% $MnSO_4 \cdot H_2O$, 0.00014% $ZnSO_4 \cdot 7H_2O$, and 0.00037% $CoCl_2 \cdot 6H_2O$. An organic nitrogen source, 0.2% proteone peptone, was used in addition to ammonium when indicated. A total of 10^7 *T. reesei* QM9414 spores, collected from cultures grown on glyceral and stored in 20% glycerol at -70°C, were inoculated into 50 ml of the medium in a 250-ml conical flask and grown at 28°C with shaking at 200 rpm. Four parallel flasks were inoculated for each medium and time point (14 to 92 h); the growth of the fungus, the pH, and the sugar consumption were analyzed; and RNA was extracted.

Cultivation on cellulose-containing medium was done under the conditions described above. The fungus was grown for 49 h in minimal medium supplemented with 1% Solka floc cellulose and 0.2% peptone. At that time, glyceral was added to 2%, and as a control, Solka floc cellulose was added to 3% final concentration, and the incubation was continued for 15 h before the mycelia were harvested. Additional control mycelia were grown for 72 h on 3% Solka floc cellulose and for 19 h on 1% Solka floc cellulose.

The cultivations in which sophorose was used as an inducer were performed as follows. A total of 10^7 spores were inoculated into 50 ml of a culture medium containing either 5% glucose, 2% sorbitol, or 2% glyceral and grown for 57 h (glucose) or 72 h (glyceral or sorbitol); 1 mM sophorose was added at that time and 10 h later, and mycelia were harvested 15 h after the first addition. To monitor the time course of *cbb1* mRNA accumulation, mycelia were harvested 3, 10, 24, and 48 h after addition of 1 mM sophorose to sorbitol cultures.

In the experiment designed to study the effect of nitrogen or carbon starvation, the fungus was grown in 250 ml of MM supplemented initially with 3% of glyceral in 2-liter shake flasks. At 48 h after inoculation, mycelia from six parallel flasks were collected, washed, and transferred into three types of fresh media: medium similar to that used from the beginning, medium lacking the carbon source, and medium lacking the ammonium sulfate present normally in MM as the nitrogen source. The incubation was continued for a further 3 days. Samples for RNA isolation, determination of mycelial dry weight, pH, and ammonia and glucose amounts in the culture medium were taken 24, 36, and 48 h after the inoculation and 2, 5, 10, 24, 48, and 72 h after the transfer to the fresh media.

Bioreactor cultivations. The bioreactor cultivation on glucose was performed in a medium initially containing 6% glucose, 0.1% yeast extract, 0.5% Bacto-Peptone, 0.4% KH_2PO_4 , 0.4% $(NH_4)_2SO_4$, 0.05% $MgSO_4$, 0.05% $CuCl_2$, 0.0005% $FeSO_4 \cdot 7H_2O$, 0.00016% $MnSO_4 \cdot H_2O$, 0.00014% $ZnSO_4 \cdot 7H_2O$, and 0.00037% $CoCl_2 \cdot 6H_2O$ in a 10-liter volume in a Chemap CF 2000 bioreactor at 29°C. Aeration was at 30 liters/min, and the pH was kept above 4.0 by automatic addition of NH₄OH. The bioreactor was inoculated with mycelia grown in glucose medium in shake flasks for 24 h in a 1-liter volume, and the glucose concentration in the inoculum was 0.8% at the time of inoculation. At 28 h of cultivation, additional yeast extract (5 g/liter) and Bacto-Peptone (5 g/liter) were added. Also, 12% glucose was fed to the culture to keep the level of glucose above 2% between 30 and 50 h of cultivation. The total amount of glucose given during this period was 465 g. After that, the fungus was allowed to consume the remaining glucose from the medium. The total cultivation time was 144 h. The consumption of glucose and the growth of the fungus were monitored, and RNA was extracted at the time points indicated in Fig. 5.

The bioreactor cultivation on cellulose was performed in a 15-liter volume in a Chemap CF 2000 bioreactor in the same medium as the glucose cultivation, except that glucose was replaced by 2% Solka floc cellulose at 29°C for 24 h and thereafter at 33°C. Aeration was at 30 liters/min. The initial pH was 5.2 without adjustment and was kept above 4.0 by automatic addition of NH₄OH. The inoculum, 1 liter, was grown in the same medium for 24 h. The cultivation was continued for 67 h, and samples for RNA isolation were taken during growth at 20, 24, 29, 42, and 67 h.

Analysis of growth and consumption of sugars and ammonia. The growth of the fungus in the 250-ml shake flasks was measured by centrifuging the contents of one flask followed by lyophilization overnight. From the bioreactor cultivation on glucose medium and the 2-liter shake flasks, samples of 50 ml (15 ml) were filtered, washed, and baked at 100°C overnight.

The sugars left in the culture media were analyzed by high-pressure liquid chromatography (HPLC). The glucose concentration in the bioreactor cultivation was measured with the GOD-Perid test kit (Boehringer Mannheim). The ammonium concentration was measured with the ammonia test combination (Boehringer Mannheim).

Northern blot analyses. Total RNA was extracted from the mycelia as described previously (7). RNA was glycosylated (47), run in a 1% agarose gel, and transferred to Hybond N nylon filters (Amersham). The hybridizations were performed in a hybridization solution containing 50% formamide, 1 M NaCl, 1% sodium dodecyl sulfate (SDS), 10% denatured sulfate, 100 µg of bovine sperm DNA per ml, and 2 to 5 $\times 10^6$ cpm of the ³²P-labelled DNA probes, which were either full-length cDNA (*cbb1*, *cbb2*, and *egf1*) or gene fragments (*cbb1*, 660 bp; *cbb2*, 660 bp; *egf1*, 660 bp; *egf2*, 820 bp; and *act*, 680 bp) probes. The RNA amount loaded on the gel was controlled by scirid orange staining. Hybridization signals (see Fig. 2 and 3) were quantified with a PhosphorImager (Molecular Dynamics), and cellulase signals were normalized to actin levels for comparisons.

Slot blot analysis of RNA. Total RNAs were diluted into appropriate concentrations with water and glycosylated by adding the same volume of the glycosylation mixture (44). After incubation for 1 h at 50°C, a dilution series was made and the samples were applied to a Hybond N nylon filter by using the Bio-Dot SF Biotinylation apparatus (Bio-Rad) as specified by the manufacturer. Filters were hybridized as described for the Northern blot analyses. The probe fragments were amplified by PCR with specific primers to obtain fragments of equal length from each gene, and these were labelled to a similar specific activity (3×10^6 cpm/µg of DNA).

RESULTS

Induction of cellulase expression. The media usually used to promote high cellulase expression are based on mixtures of plant materials or cellulose. These materials interfere with the estimation of dry weight and isolation of RNA and are also often undefined in nature. For these reasons, the first shake flask experiments were carried out on medium containing 2% cellobiose as the only carbon source, and the results were compared to those obtained with 2% glucose cultures. Mycelial dry weight, pH, carbon source utilization, and expression of the glycolytic phosphoglycerate kinase gene, *pck1*, as a general control of metabolism (62) were analyzed regularly from the cultures.

Cellobiose clearly induced the expression of *cbb1* (Fig. 1A) and, in a similar manner, that of the other cellulase genes (data not shown) after a rather long lag period. Reduced expression was observed at a later stage of growth, when glucose was accumulating although cellobiose was still present. No cellulase transcripts were detected in similar cultures on glucose-containing media (Fig. 1C). The presence of an organic nitrogen source has been suggested to be important for cellulase production on Solka floc cellulose medium (25), and the effect of addition of peptone to cellobiose cultures was analyzed. This did not improve expression (Fig. 1B); on the contrary, expression was barely detectable. This result indicates that peptone seems not to enhance induction per se, and the earlier reports on its positive effect on cellulase production could instead be due to increased growth of the fungus and subsequent elevated cellulase levels in production conditions.

It has been suggested previously (see Discussion) that cellulase expression would occur on carbon sources supporting only poor growth. The expression patterns observed in the cellobiose cultures would be in accordance with this: lower expression was obtained in the cellobiose-peptone culture, where growth was clearly more rapid than on the cellobiose medium in which ammonium was the only nitrogen source. However, no expression occurred in a similar shake flask experiment carried out on 2% sorbitol medium (Fig. 2, lanes 2 and 3; Fig. 3, lane 1), where growth was also poor, even poorer than on cellobiose (data not shown).

Additional studies showed that sorbitol may be used as a nonrepressing, noninducing carbon source. As mentioned above, no expression occurred on sorbitol medium but addition of sophorose to the culture medium efficiently induced cellulase expression (Fig. 2, lanes 2 to 4). Sophorose (1 mM) was added twice to the sorbitol culture, at 72 and 82 h, and mRNA analyses were performed 15 h after the first addition, when over 1 mM sophorose was still present in the medium.

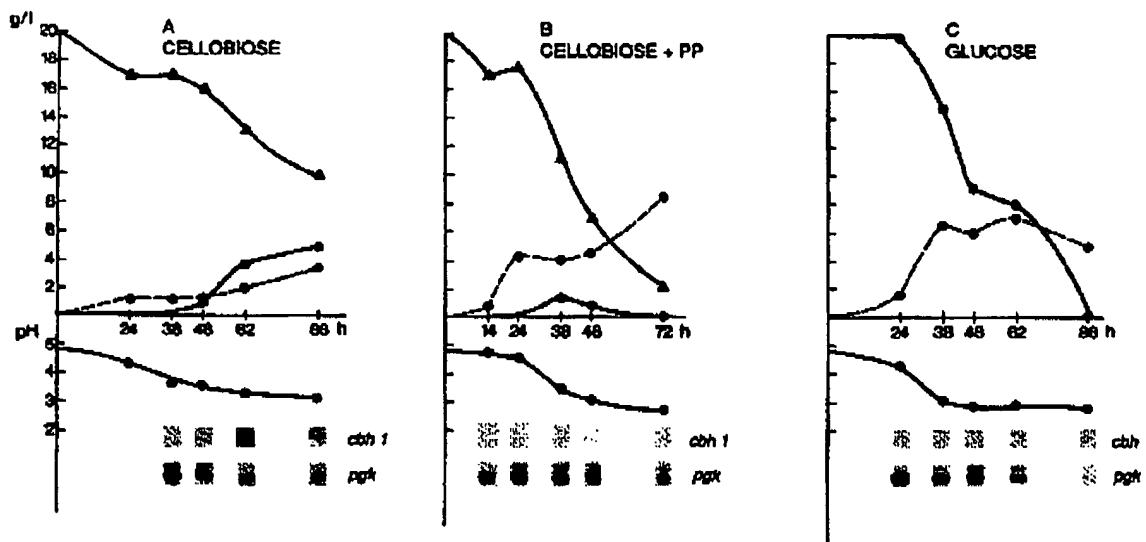


FIG. 1. Shake flask cultivations on media containing 2% cellobiose (A), 2% cellobiose plus protease peptone (PP) (B), and 2% glucose (C). The amounts of cellobiose (Δ) and glucose (\bullet), the fungal dry weight (\square), and pH of the medium (\circ) were analyzed and samples for RNA isolations were taken at the time points indicated. Northern analyses of total RNA (3 μ g) using *cbh1* and *pgk* probes is shown below.

Strong sophorose induction of the cellulases *cbh1* and *cbh2* was observed, and *egf1*, *egf2*, and *egf5* were also induced, although less efficiently. No mRNAs could be detected at different stages of growth without addition of the inducer (Fig. 2, lanes 2 and 3). Also, the presence of 2% cellobiose in sorbitol cultures from the beginning of the cultivation provoked some cellulase transcription (Fig. 2, lane 1), and this level was comparable (data not shown) to that obtained in the cultivations carried out on cellobiose alone (Fig. 1).

That prolonged strong sophorose induction of cellulases can be obtained by adding small amounts of sophorose to sorbitol cultures under the above-mentioned conditions was further shown in a separate set of experiments in which the time course of sophorose induction was investigated (Fig. 3). Moderate *cbh1*, *cbh2*, *egf1*, and *egf5* expression was detected 3 h after the addition of sophorose, and the mRNAs continued to accumulate and remained abundant until at least 48 h. Sophorose induction of *egf2* was observed in a longer exposure. These steady-state mRNA levels are comparable to those obtained when Solka floc cellulose is used as the sole carbon source as shown in Fig. 2 (lanes 4 and 10 to 12).

The finding that sorbitol can in practice be considered a neutral carbon source for cellulase expression prompted us to study the nature of glycerol in this respect. Sophorose was added twice to 2% glycerol cultures in a similar manner to that described above for the sorbitol cultures. A high level of cellulase expression occurred; the data for the five cellulases are shown in Fig. 2, lanes 5 to 9. Sophorose induction of cellulases was observed in cultures containing 5% sorbitol or glycerol as well (data not shown). Thus, both sorbitol and glycerol can be considered "neutral" carbon sources with respect to cellulase expression in contrast to glucose (see below).

The comparison between the RNA samples collected from the different growth conditions presented in Fig. 2 revealed that the highest expression levels of all the cellulase genes studied were reached in glycerol-sophorose cultures, closely

followed by Solka floc cellulose and sorbitol-sophorose (Fig. 2, lanes 4 and 8 to 12). The differences between these highly induced conditions were two- to fourfold. In the sorbitol-cellobiose cultures (Fig. 2, lane 1), the expression levels were about 30-fold lower than under the highly induced conditions.

Relative expression levels of the cellulases. To study in more detail the relative transcript amounts of the cellulases *cbh1*, *cbh2*, and *egf1*, which are considered the three major cellulases, RNAs from mycelia grown under different inducing conditions were compared by a slot blot analysis with probes of the same length and specific activity. Figure 4 shows the results from the shake flask cultivations carried out on sorbitol-sophorose medium and on 3% lactose medium. Also, samples from a bioreactor cultivation on Solka floc cellulose containing medium were included in the analysis. The steady-state mRNA levels of *cbh1* were the highest of the cellulases under all conditions, followed by *cbh2* and *egf1*. *cbh1* mRNA was approximately 1.5 and 3 times more abundant than *cbh2* and *egf1* mRNAs, respectively, as determined by laser scanning densitometry, although the visually estimated differences seemed larger (Fig. 4). The mRNA levels on lactose medium remained clearly lower than on sorbitol-sophorose and cellulose media, with the difference in steady-state mRNA levels between lactose and cellulose being about 60-fold. In a separate slot blot experiment, the expression level obtained on the 2% cellobiose medium was shown to be comparable to that of the lactose culture (data not shown). This is well in accordance with the 20-fold difference reported at the protein level between cellulose and cellobiose cultures (27) and the estimations presented above based on results in Fig. 2. It is noteworthy that as observed in the shake flask experiments (Fig. 2), the induction levels after 15 h of sophorose induction approach those obtained on Solka floc cellulose medium after 20 h in a bioreactor.

In the bioreactor cultivation on Solka floc cellulose medium, the cellulase transcript levels were clearly higher than the mRNA levels of the phosphoglycerate kinase gene, *pgk1*, in-

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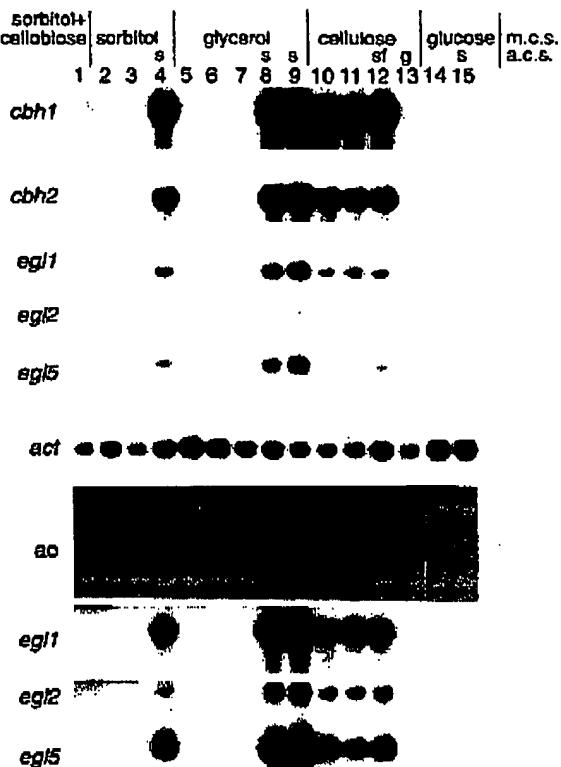
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FIG. 2. Induction of *cbh1*, *cbh2*, *egl1*, *egl2*, and *egl5* expression by sorghose and cellulase in cultures containing sorbitol and glycerol and repression in the presence of glucose. RNA was isolated from mycelia grown on media containing 2% sorbitol plus 2% cellulase (lane 1), 2% sorbitol (lanes 2 to 4), 2% glycerol (lanes 5 to 9), Salka fine cellulase (lanes 10 to 13), or 5% glucose as the main carbon source (m.c.s.). Additional carbon sources (a.c.s.) were added to selected cultures as follows: 1 mM sorghose (s) was added to sorbitol and glycerol cultures at 72 and 82 h and to glucose cultures at 57 and 67 h. Mycelia were collected 15 h after the first addition. Glucose (g) was added to 2% and Salka fine cellulase (sf) was added to 5% final concentration at 49 h of the cellulose-based cultivation, and the mycelia were collected 15 h later. A 5- μ g portion of total RNA was loaded onto the gel. Four identical blots were prepared and hybridized simultaneously with the cellulase probes, *cbh1*, *cbh2*, and *egl1* probes were of the same length and specific activity (1×10^6 cpm/ μ g); *egl2* and *egl5* probes were less active (2×10^6 cpm/ μ g). Control hybridizations with an actin probe (*act*) were done after stripping the membranes, and the result is shown for one of the gels together with the acridine orange staining (ao). The filters were exposed for the same time (four top panels). Longer exposures of the endoglucanase hybridizations are shown in the two lowest panels. Lanes: 1, sorbitol plus cellulase, 72 h; 2, sorbitol, 72 h; 3, sorbitol, 87 h; 4, sorbitol plus sorghose, 87 h; 5, glycerol, 72 h; 6, glycerol, 87 h; 7, glycerol, 92 h; 8, glycerol plus sorghose, 87 h; 9, glycerol plus sorghose, 92 h; 10, 5% Salka fine cellulase, 72 h; 11, 1% Salka fine cellulase, 49 h; 12, 1% plus 2% Salka fine cellulase, 64 h; 13, 1% Salka fine cellulase plus 2% glucose; 14, 5% glucose, 72 h; 15, glucose plus sorghose.

volved in glycolysis, with the difference between *cbh1* and *pck1* being roughly 50-fold. On the other hand, on cellulose medium, where induction of cellulases is weaker, the steady-state expression level of *pck1* exceeded that of the cellulases (data not shown).

Glucose repression of cellulase expression. Data from the shake flask cultivations showed that glucose acts as a repressor of cellulase synthesis at the transcriptional level. No transcript-

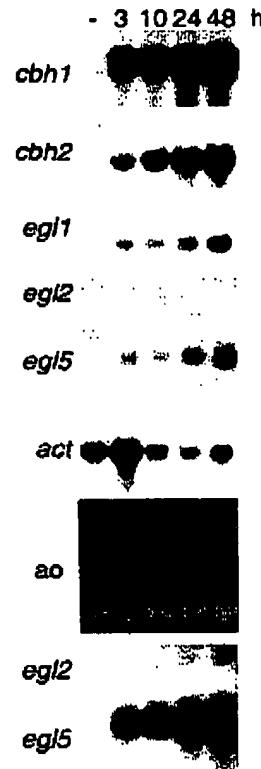


FIG. 3. Time course of *cbh1*, *cbh2*, *egl1*, *egl2*, and *egl5* mRNA accumulation after sorghose addition to 2% sorbitol cultures. Incubation was carried out for 72 h on sorbitol; then 1 mM sorghose was added, and RNA was isolated at the time points indicated after sorghose addition (3, 10, 24, and 48 h) and before sorghose addition (—). Northern blot analysis was performed exactly as described in the legend to Fig. 2 and hybridized with the probes indicated on the left. The filters were exposed for the same time (four top panels). A longer exposure is included to demonstrate the *egl2* signal (lowest panel).

tion was observed if sorghose was added to a glucose-based culture (Fig. 2, lanes 14 and 15). The repressing action of glucose on cellulase expression was further shown by addition of glucose to fully induced cellulose-based cultures (lanes 10 to 13), which resulted in the disappearance of the cellulase transcripts.

To address further the question of possible low basal levels of expression on pure glucose, *T. reesei* QM9414 was grown in a bioreactor (Fig. 5). This allows the glucose level to be carefully controlled and several RNA samples to be analyzed at different time points of the culture. No signals with the cellulase probes *cbh1*, *cbh2*, and *egl1* were detected in Northern blot analyses at different time points when glucose was present in the culture (Fig. 6, time points 35 to 75 h). However, the presence of low constitutive expression levels cannot be necessarily excluded on the basis of a regular Northern blot analysis, and for this reason a sample from the 35-h time point was included in the same dot blot analysis as described above (Fig. 4). No cellulase transcripts were detectable, even when overloading the analysis with RNA. Comparison of the different culture conditions shows that the steady-state cellulase transcripts would be at least 1,000-fold (in the case of *cbh1*, roughly

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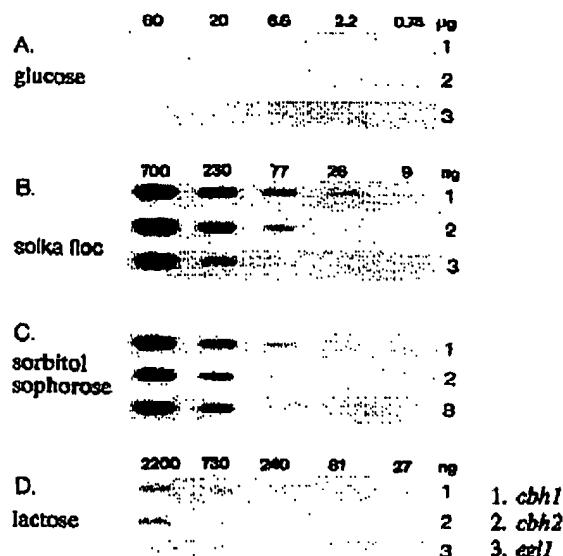


FIG. 4. Slot blot analysis on steady-state cellulase transcript levels produced under various culture conditions. Total RNA (0.74 to 60 µg) from glucose-grown mycelia (A) (bioreactor cultivation, 35 h), total RNA (9 to 700 ng) from Solka Floc cellulose-grown (B) (bioreactor cultivation, 20 h) and sorbitol-sophorose grown (C) (shake flask cultivation, 15 h after sophorose addition; total time, 87 h), mycelia, and total RNA (27 to 2200 ng) from lactose-grown mycelia (D) (shake flask cultivation, 96 h) were loaded. Cellulase probes of equal length were labelled to the same specific activities and are indicated on the right by numbers: 1. *cbh1*; 2. *cbh2*; 3. *egl1*.

6,000-fold) more abundant in the bioreactor cultivation on Solka Floc cellulose medium than in the bioreactor cultivation on glucose, provided that expression occurred on glucose but was not detectable by the method applied. Addition of an inducer did not change the situation, since no mRNA was

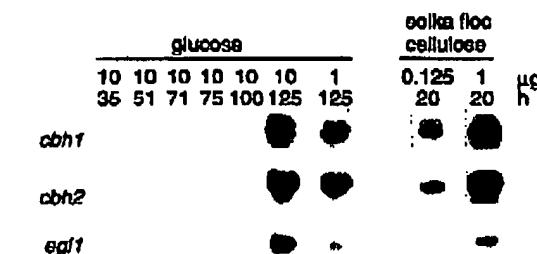


FIG. 6. Northern blot analyses of expression of the cellulase genes *cbh1*, *cbh2*, and *egl1* during bioreactor cultivation of glucose-containing medium at different time points. For comparison, samples from a Solka Floc cellulose cultivation in a bioreactor (20 h) are shown on the right. Total RNA at 0.125 to 10 µg was loaded onto the gel as indicated above each lane.

detected when 60 µg of RNA from the previously described 5% glucose-sophorose shake flask cultivation (Fig. 2, lane 15) was analyzed in a similar dot blot analysis (data not shown). These data show that glucose repression of cellulase expression is strong in *T. reesei* QM9414. No cellulase mRNAs were observed when RNAs were analyzed similarly from the fungus grown on sorbitol medium (data not shown).

Analyses of multiple RNA samples from glucose-grown mycelia have always produced negative results with *cbh1*, *cbh2*, *egl1*, and *egl2* probes. This has usually been the case with *egl5* hybridizations, except that in a few cases a positive signal has been detected (data not shown), and the significance of this observation should be studied further.

The fungus was allowed to consume the glucose completely in the bioreactor cultivation described above (Fig. 5), and RNA samples were also analyzed from this period. Surprisingly, after a lag period of roughly 45 h after glucose depletion from the culture, relatively high levels of *cbh1*, *cbh2*, and *egl1* mRNAs were detected in the Northern analyses (Fig. 6). The mRNAs were already slightly visible at the 100-h time point, although they were not detectable with the exposure time shown in Fig. 6. At the 125-h time point, the expression levels

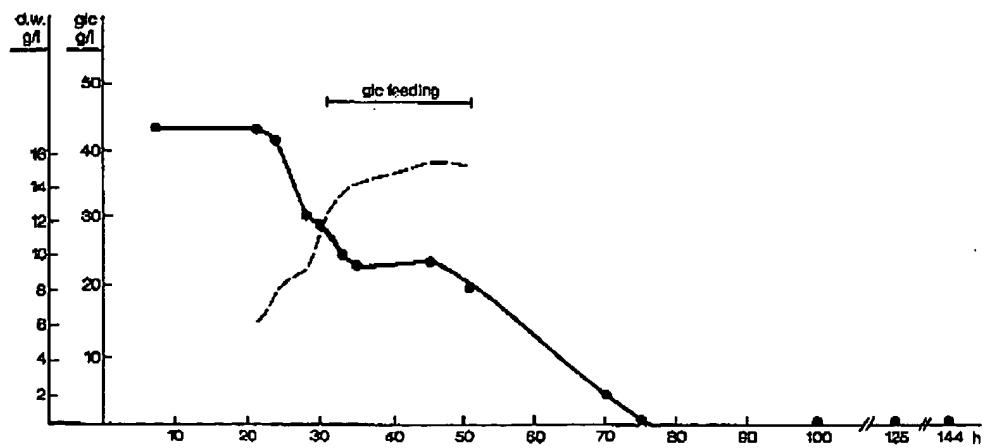


FIG. 5. Bioreactor cultivation on glucose-containing medium. The glucose concentration in the medium (solid line) and the dry weight of fungus (dashed line) were measured, and RNA was isolated at the time points indicated by dots. Concentrated glucose solution was fed into the bioreactor between 30 and 50 h of cultivation, after which the fungus was allowed to consume all the glucose from the medium.

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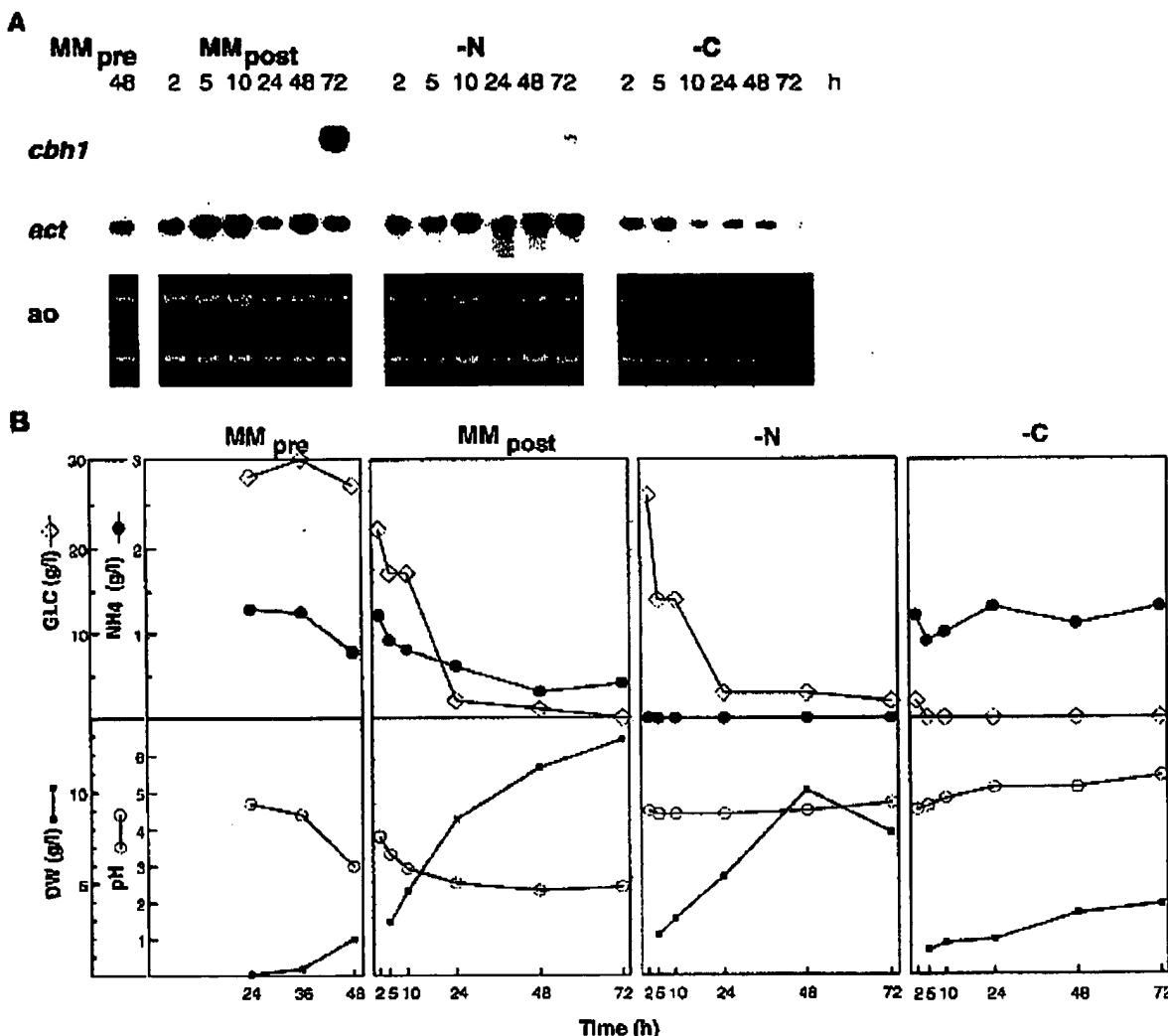
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FIG. 7. Northern analysis on *cbh1* expression (A) and courses of the shake flask cultivations (B) on minimal medium and on media lacking either a nitrogen or a carbon source. The fungus was grown for 48 h in minimal medium containing 24 g of glucose per liter and 1.3 g of ammonia per liter (MM_{pre}). Thereafter, the mycelia were harvested, washed, and divided into fresh minimal medium supplemented with both glucose and ammonia (MM_{post}) and into minimal medium lacking nitrogen (-N) or carbon (-C), and the incubation was continued for a further 72 h. Amounts of glucose (Glc) and ammonia, pH, of the medium, and mycelial dry weight (DW) were determined, and RNA was isolated at the time points indicated. A 2-μg portion of RNA was loaded onto the gel. Hybridization to an actin probe (*act*) is shown as a control. An acridine orange-stained gel.

were roughly 10% of those detected in the bioractor cultivation on the cellulose medium shown for comparison. This result demonstrates that cellulase expression can be obtained after glucose depletion without an apparent addition of an inducing compound. We had earlier observed that this kind of derepression also occurs in prolonged shake flask cultivations on 2% glucose medium, especially if peptone is included, occurring then after about 24 h of glucose depletion (data not shown).

To further study if carbon or, alternatively, nitrogen depletion as such is provoking cellulase expression, the fungus was grown in shake flasks in a normal minimal medium supple-

mented with glucose and containing ammonium sulfate as a nitrogen source. In an active growth phase, the mycelia were washed and transferred into three different media, one the same as that used from the beginning (to serve as a control), one depleted of carbon, and one depleted of nitrogen (Fig. 7). Expression of *cbh1* was not detected when analyzed at different time points between 2 h and 2 days after the transfer. Only 3 days after the transfer, *cbh1* mRNA was produced in the control cultures containing glucose and ammonia or in the culture containing glucose but depleted of nitrogen (Fig. 7A). This coincided with very low glucose levels in the medium. Expression of *cbh1* was not detected in the culture transferred to a

medium lacking the carbon source (Fig. 7A), although, for instance, the starvation-inducible hydrophobin mRNA has already appeared after 2 to 10 h in the same experiment (34). The results obtained with *cbh2* and *egf1* probes (data not shown) were identical to *cbh1*. The RNA yield was very low at the late stages of the experiment in the medium without a carbon source (Fig. 7B), indicating that there were only few viable cells, which made it impossible to continue the experiment any further. These results suggest that carbon starvation per se is not sufficient to initiate cellulase expression, because complete removal of the carbon source from the medium had no effect even after 3 days of incubation.

DISCUSSION

Cellulose and sophorose exerted the highest levels of cellulase transcription in *T. reesei* QM9414, followed by moderate levels on lactose and cellobiose and no expression on glucose, glycerol, or sorbitol. The results give a general picture of the potential of cellulase expression on these different carbon sources and are in accordance with cellulase protein levels generally obtained. The relative transcript levels of the different cellulase genes with that of *cbh1* being the highest, are also comparable to the amounts of the specific proteins produced in the culture medium (16). It was suggested previously (19, 27) that cellulase expression would not occur on carbon sources that promoted rapid growth, such as glucose and glycerol, but would be provoked on poor carbon sources, such as cellulose, cellobiose, and lactose. Our results do not show correlations between growth and cellulase expression, a conclusion also reached by others (31). Rather, our results show that distinct mechanisms of induction and repression are operating in the cellulase expression in *Trichoderma*.

The results clearly show the strong inducing power of sophorose when added at 1 to 2 mM to the culture; this was studied at the mRNA level previously (10, 12). Sophorose is consequently a good candidate for the natural inducing compound for cellulase expression. The reports on the use of cellobiose as a carbon source for cellulase expression are, on the other hand, to some extent controversial. Cellobiose has been considered a poor inducer (13, 15, 16, 37, 43) but is also to exert as good induction as cellulose if growth is kept slow and the level of cellobiose is kept low by controlled feeding of cellobiose into the culture (59). In the cultivations presented here, cellobiose promoted cellulase transcription to a moderate level comparable to that of lactose, which shows the inducing effect of cellobiose or of sophorose formed from it. The difficulty with this carbon source is, however, evident, as induction is not immediate and seems to be variable depending on the culture conditions. The failure to provoke cellulase expression could be explained, on one hand, by the lack of an inducing effect when consumed rapidly from the culture, as observed in this study in the presence of peptone, or, on the other hand, by the accumulation of glucose and subsequent glucose repression of cellulase transcription. The inducing power could thus be dependent on the ratio between sophorose and glucose formation by β -glucosidase and their uptake from the medium. It is also likely that organic nitrogen sources such as peptone are used as both nitrogen and carbon sources (see also reference 21) and could somehow interfere with cellulase expression.

As reported previously (10), no cellulase mRNAs could be detected in fungus grown on glycerol as the only carbon source. However, glycerol has not been previously studied at the molecular level in the presence of an inducer, and the literature available groups this carbon source with glucose as being a

compound inhibiting cellulase expression (see reference 21 for discussion). Our results clearly show that glycerol, as well as sorbitol, does not as such promote expression but does not inhibit it either, because addition of sophorose to glycerol or sorbitol cultures provokes high cellulase transcript levels. Thus, the cellular effects exerted by glucose and glycerol-sorbitol are clearly distinct. This is in agreement with the situation in the yeast *Saccharomyces cerevisiae* (14). Studies on cellulase regulation are very much hampered by the fact that the most natural carbon sources promoting cellulase expression, such as cellulose, are also used for growth by the fungus. The use of glycerol and sorbitol as carbon sources now allows powerful and rapid cellulase gene induction to be obtained in growing cultures with 1 to 2 mM sophorose without significantly affecting the growth of the fungus.

It is generally believed that small soluble molecules are released from cellulose and act as inducing compounds or are converted to such. To explain this initial attack, cellulase expression has been suggested to occur constitutively at low levels under all conditions including only glucose and no inducer as a carbon source, and evidence for this has been presented (10, 20, 22, 31). The analysis carried out in this work shows that glucose repression is tight, and the differences in mRNA levels between fully induced and repressed states are great in an actively growing fungus provided that the presence of glucose in the culture is carefully controlled. It is still possible, however, that biologically relevant mRNA levels remain undetected in experiments based on steady-state mRNA levels if the mRNA turnover on glucose-based media is especially rapid. Mechanisms of glucose repression are in any case operating in the regulation of cellulase expression. We have recently shown by site-specific mutagenesis of the *cbh1* promoter that regions similar to the binding sites of the regulatory proteins MIG1 of *S. cerevisiae* (35) and CREA of *Aspergillus nidulans* (24), involved in glucose repression in these organisms, seem to be at least partially responsible in vivo for the glucose repression of *cbh1* (18). The *Trichoderma* equivalent of *creA*, *cre1*, has recently been cloned (17, 52, 53). The demonstration that cellulase expression occurs on glucose-containing medium in the hypercellulolytic *T. reesei* strain Rut-C30 and that this is caused by a mutant form of the *cre1* gene (17) further confirms glucose repression of cellulase expression. The earlier reports pointing toward constitutive expression on pure glucose could be explained by the use of the mutant strain Rut-C30 in the studies or, alternatively, by the release of glucose repression once glucose has been consumed from the medium. This kind of derepression was clearly seen in both shake flask and bioreactor cultivations carried out in this work.

This capability of cellulase expression after glucose depletion, without the addition of an inducer, could provide the fungus with a survival mechanism under starvation conditions. Starvation has previously been suggested to induce cellulase synthesis (15). Interestingly, however, depletion of glucose as such was clearly not enough to initiate cellulase expression, as demonstrated by a prolonged incubation of pregrown washed mycelium in a medium containing no carbon source. This suggests that some kind of induction is required, and this might be related to growth since cellulase expression occurred in cultures where growth had occurred (Fig. 7). It is possible that oligosaccharide molecules released from the cell walls of the starving fungus act as inducing compounds or are converted to them. It is also possible that sophorose or some other inducing sugar is formed from the glucose present in the culture by the transglycosylation activity of the possible constitutive β -glucosidase (51, 57) and will later exert induction after glucose has been depleted and repression released. The latter alternative is

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supported by the fact that cellulase expression was not detected in mycelia incubated without a carbon source but was detected in glucose-grown mycelia (starved or not starved for nitrogen) after glucose was depleted from the medium. However, no sophorose could be detected in the medium in the glucose-based bioreactor cultivation by HPLC, which indicates that if extracellular sophorose was present, its level was below the detection limit, 30 µM. Conidium-bound cellulases (22, 32) could provide another means for the fungus to attack cellulose in the absence of an inducer. No indication for conidiation was, however, observed at the later stages of the cultivations presented in this work.

Earlier mutant data suggest that separate regulation could exist for β -glucosidases, cellobiohydrolases, and endoglucanases (9, 33, 36, 48, 49). Our data show that the major cellulases, cellobiohydrolases I and II and the endoglucanases I and II of *T. reesei*, are coordinately expressed under all the conditions studied. This also seems to be the case in the derepressed stage after glucose depletion. These results indicate a common regulatory mechanism which retains the proportions of these cellulases. This, however, does not contradict the possibility that specific regulatory mechanisms are revealed in a mutant situation which, in the wild-type situation, all contribute to the overall control, keeping it apparently similar for all cellulases under standard conditions. Furthermore, *T. reesei* still produces at least one other β -glucosidase (6) and an additional endoglucanase, EGIII (63). Consequently, it is still possible that these cellulases, EGV, or some yet unknown enzymes, escape common regulation and are more constitutively expressed, thus having the potential of immediate release of an inducer once cellulose is present.

ACKNOWLEDGMENTS

The first two authors contributed equally to this work.

The technical assistance of Seija Nordberg and Riitta Nurmi is warmly appreciated. Michael Bailey is thanked for the bioreactor cultivations. Tiina Nakari-Seitila is thanked for certain RNA samples, and Sirkka Keränen is acknowledged for useful discussions. Hannale Virtanen and Marjukka Perttilä are thanked for the HPLC analyses, and Olli Lappalainen is thanked for secretarial work.

Financial support from Alko Ltd. and the Foundation for Biotechnical and Industrial Fermentation Research (supported by Alko Ltd.) is gratefully acknowledged.

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